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CONTRACT NUMBER DAMD17-95-C-5001

TITLE: The Molecular Biological Basis for the Response of Poly(ADP-RIB) Polymerase and NAD Metabolism to DNA Damage Caused by Mustard Alkylating Agents

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REPORT DATE: July 1996

TYPE OF REPORT: Midterm

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1996	Midterm (1 Jan 95 - 30 Jun 96)

4. TITLE AND SUBTITLE
The Molecular Biological Basis for the Response of Poly(ADP-RIB) Polymerase and NAD Metabolism to DNA Damage Caused by Mustard Alkylating Agents

5. FUNDING NUMBERS
DAMD17-95-C-5001

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

During the course of this contract, we have performed a variety of experiments to provide a strategy to modulate the nuclear enzyme poly(ADP-ribose) polymerase (PARP), in cultured keratinocytes. This enzyme modifies a variety of nuclear proteins utilizing NAD. DNA is required for the catalytic activity of the enzyme and the activity is dependent upon the presence of strand breaks in this DNA. It has been hypothesized that human skin exposed to mustards may develop blisters due to a generalized lowering of NAD in exposed skin cells.

During the contract period, we have established a stably transfected human keratinocyte cell line which expresses antisense transcripts to PARP mRNA when these keratinocyte were grafted onto nude mice they formed histologically normal human skin. Accordingly, a model system has been developed in which the levels of PARP can be selectively manipulated in human keratinocytes in reconstituted epidermis as well. We also showed that PARP was proteolytically cleaved at the onset of spontaneous apoptosis following proteolytic conversion of CPP32b to its active form, termed "apopain". Having characterized the events associated with apoptosis, we determined, during the last period, whether any or all of these features could be observed following exposure of keratinocytes to SM.

14. SUBJECT TERMS	15. NUMBER OF PAGES
RA V; Human Tissue; Skin; Lab Animals; Cell Culture; Athymic Mice; Sulfur Mustard; Poly(ADP-Ribose); apoptosis	33
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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PI - Signature Date

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INTRODUCTION

A marked decrease in the cellular content of NAD occurs in response to DNA strand-breaking agents such as sulfur mustards. This response, which depletes the cell of energy reflects the synthesis of poly(ADP-ribose), reserves and, ultimately, by activating proteases, is thought to contribute to the formation of blisters in subcutaneous areas of skin [2]. e are testing aspects of this hypotheses by developing genetic methods to modulate experimentally poly(ADP-ribose) metabolism in human skin cells. Understanding the basic mechanism of this process may allow the development of treatments to reduce the pathological effects of such chemical warfare agents on skin as well as the identification of molecular markers for assessment of exposure to subsymptomatic doses of these agents.

Strand breaks in DNA greatly stimulate the synthesis of poly(ADP-ribose) in the nuclei of all eukaryotic cells. Because the substrate for poly(ADP-ribose) polymerase (PARP), the enzyme that synthesizes poly(ADP-ribose), is NAD, the DNA strand breaks induced by exposure of skin cells to chemical warfare agents result in a marked depletion of NAD. Papirmeister *et al.* [2] have proposed that such NAD depletion in skin cells results in inhibition of glycolysis and that the consequent accumulation of metabolic intermediates stimulates the NADP-dependent hexose monophosphate shunt and both the synthesis and extracellular release of proteases. These proteases may be responsible for development of subepidermal blisters. Although this biochemical scenario has been confirmed in part, the studies underway in the current program aim to further substantiate this hypothesis with the use of recombinant DNA techniques to modulate PARP expression in skin cells.

Nuclear poly(ADP-ribosyl)ation is a key defense of cells against DNA-damaging agents such as alkylating drugs, sulfur mustards, and other agents to which the skin of military personnel may be exposed. Sulfur mustards exhibit cytotoxic, carcinogenic, and vesicant properties. Mustard gas induces alkylation of purine bases in DNA which is ultimately repaired by endonucleases that cleave the phosphodiester bonds of DNA. Our laboratory as one of the first to show that such DNA strand breaks activate PARP.

Our studies directly address the effects of sulfur mustard agents on the poly(ADP-ribose) content of skin with the use of human keratinocytes transfected with an inducible expression vector that encodes PARP antisense RNA. After establishment and characterization, these cells have been grafted to nude mice, in which they are maintained as a layer of human skin.

In addition to chemical carcinogens, PARP is activated by ultraviolet and ionizing radiation. Thus, PARP is likely to play an important role in the repair of DNA within the epidermis. The localization of PARP to cells of the lower layers of the epidermis, including the basal or proliferating cells, which are more susceptible to the effects of DNA-damaging agents, is consistent with this notion [1]. The role of PARP in mediating the biological response to DNA strand breaks is also supported by studies that show that the increase in PARP activity and the consequent decrease in the amount of intracellular NAD (and, hence, ATP), may be responsible for or contribute to the blistering response of individuals exposed to sulfur mustards, and possibly to other DNA strand-breaking agents [2-7]. In addition to its role in DNA repair, studies implicate PARP in both cellular proliferation and differentiation [8, 9].

The role of PARP in epidermal differentiation is of particular interest to us, because the progression of dividing basal cells to mature cornified envelopes comprises a relatively well-defined sequence of biochemical and physiological events. Furthermore, perturbations in this process can lead to a variety of skin lesions, including squamous cell and basal cell carcinoma.

MIDTERM RESULTS ON KERATINOCYTES AND PARP ANTISENSE RNA

Cell Culture, Transfection, and Grafting. Human keratinocytes, a kind gift from R. Schlegel, were derived from NHEK cells immortalized with the Neo I fragment of human papilloma virus (HPV) 18 containing the coding regions for E6 and E7 [10]. Keratinocytes were grown in KGM + DMEM (3:1) medium containing 2.5% fetal bovine serum (FBS). Cells were grown to 80% confluency and split 1:5. Cells transfected by lipofection with the BRL (Bethesda, MD) lipofection reagent. Colonies ere selected by resistance to the drug G418 (Life Technologies) at 150 mg/ml. Resistant cells were colony-isolated and grown in the presence of G418 until the colonies were large enough to passage into 60-mm culture dishes. (5×10^6) were then grafted along with 8×10^6 primary mouse fibroblasts onto the backs of nude mice accordint to the grafting chamber technique as previously described for mouse keratinocytes [11].

Plasmids and Polymerase Chain Reaction (PCR) Analysis. The construction of pMX18, in which the full-length human PARP cDNA was placed in the antisense orientation in pMAMneo (Promega) under the control of the murine mammary tumor virus (MMTV) dexamethasone-inducible promoter, has been described [12].

For PCR, cell extracts containing total genomic DNA were derived from each clone by methods described previously [13]. The 22-bp upstream primer was based on the MMTV long terminal repeat (LTR) sequence (beginning at nucleotide 981) of pMAMneo:5 prime-ACA GTG GCT GGA CTA ATA GAA C. The 23-bp downstream primer was located 771 bp from the upstream primer, within the sequence encoding the DNA-binding domain of human PARP or 3 prime GTT AGA ATG TCT GCC TTA CTG GT. PCR as performed with a Temp-Tonic thermal cycle (Thermolyne), with a denaturing step at 95°C for 15 s, annealing at 58°C for 30 s, and primer extension at 72°C for 30 s. Forty cycles of amplification ere performed, followed by a 4-min extension step at 72°C. PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide.

Antibodies and Immunocytochemical Analysis. Purified human PARP synthesized in Escherichia coli AR 58 cells was incubated with NAD in the presence of sheared DNA to induce automodification. Polymer was released from the protein by alkaline hydrolysis. Free polymer was separated by electrophoresis and polymers consisting of 20 to 40 ADP-ribose moieties were isolated and used to immunize guinea pigs. Both preimmune and immune sera were collected and analyzed for specificity by immunoblot analysis with automodified PARP. Polyclonal rabbit antiserum to human PARP was a kind gift from R. Roeder. Polyclonal rabbit antiserum to porcine PARP, which also recognizes mouse PARP, was generously supplied by H. Hilz [14].

Cells were grown on eight-ell chamber slides (Tissue-Tech). The MMTV-driven constructs were induced in culture by the addition of 1mM dexamethasone to the culture medium. After different time intervals, cells were washed twice with phosphate-buffered saline (PBS) and fixed by the addition of 10% ice-cold trichloroacetic acid (TCA). After 10 min, cells ere washed successively with 70%, 90%, and absolute ethanol for 10 min. Slides were then either processed for immunofluorescence immediately or stored at -20°C. For visualization of PARP or poly(ADP-ribose) polymer, TCA-fixed cells ere incubated overnight at room temperature in a humid chamber with antibodies to human PARP diluted 1:500 or with antibodies to poly(ADP-ribose) diluted 1:1000 in PBS containing 12% bovine serum albumin (BSA). The next day, slides were washed with PBS three times for 10 min, and then once with distilled H_2O for two min. The slides were then incubated with biotinylated antibodies to guinea pig immunoglobulin diluted 1:800 in PBS containing 12% BSA. After three PBS washes and one distilled H_2O wash as above, slides were incubated with streptavidin-Texas Red conjugate (1:400; Life Technologies) and fluorescein-conjugated antibodies to rabbit immunoglobulin (1:40; Dako) in BSA:PBS buffer. For visualization of

keratins, mouse monoclonal antibodies to keratins were used (Chemicon). Cells were again washed as above and mounted with coverslips with PBS containing 80% glycerol. Immunofluorescence as visualized with a Zeiss immunofluorescence microscope equipped with a Nikon camera.

Ribonuclease Protection Assay and *in Situ* Hybridization. Total RNA from 10^8 cells was obtained for each cell clone by the guanidinium-phenol method [15] and analyzed by ribonuclease protection assay (RPA) with ^{32}P-labeled RNA probes (5 x 10^8 to 6 x 10^8 cpm/mg of template DNA). The RNA probe used to detect the endogenous PARP mRNA was derived from the full-length human PARP cDNA cloned into the Sal I site of pGEM 4Z (Promega). The plasmid as linearized at the Eco RI site and transcribed *in vitro* with T7 polymerase. This yielded a 618-bp probe that protects a 588-bp fragment representing the 3¢ region of PARP mRNA. As an internal control, RPA was performed with a radiolabeled RNA probe derived from a riboprobe vector containing a 220-bp Pst I fragment of the PO human acid ribosomal protein [16]. This vector, a kind gift from M. Martin, was linearized with Eco RI and transcribed with T7 polymerase to generate the complementary strand to the endogenous ribosomal protein mRNA. Hybridization and RNase digestion were performed as described [17].

For *in situ* hybridization, three weeks after grafting, graft sites of live animals were treated topically with either 75 mg of dexamethasone in 75 ml of acetone, or with 75 ml of acetone alone. After 24 h, animals were killed by cervical dislocation and the grafted skin embedded in OCT, quick-frozen on dry ice, and stored at -80°C until use. *In situ* hybridization was performed as described previously [18]. For a riboprobe, the full-length human PARP cDNA cloned into the Xho I site of pGEM 4Z (Promega) was linearized at the Acc I site of PARP, and the sense strand (specific for antisense transcripts) was transcribed with the use of T7 polymerase.

To generate stable lines of keratinocytes that synthesize PARP antisense RNA in an inducible manner, it was necessary to obtain keratinocytes that were immortalized, but not transformed (i.e., that could form normal skin in the graft system; see below). Because of the results of grafting experiments, we elected to use a clone of human epidermal keratinocytes immortalized with the E6 and E7 genes of HPV 18, which formed histologically and immunocytochemically normal skin. These cells were transfected by lipofection with a plasmid containing human PARP cDNA in the antisense orientation under the control of the MMTV promoter. Following G418 selection, approximately 25 resistant clones were isolated. DNA as isolated from each of the clones and examined by PCR analysis with

primers specific for the MMTV LTR and PARP cDNA to determine whether the PARP antisense construct was stably integrated. Twenty of the clones were positive for the correct integration of the PARP cDNA with respect to the MMTV promoter,as determined by the presence of a specific 771-bp amplified fragment detected on ethidium bromide-stained agarose gels. Two stable lines (AS-1 and AS-2) containing the antisense construct were chosen for further analysis, along with control cells transfected with the vector alone.

Depletion of endogenous PARP mRNA in transfected cells--We net determined the fate of PARP mRNA following induction of PARP antisense RNA. For determination of PARP mRNA levels in the keratinocyte clones, cells were kept at subconfluent densities. A sensitive RPA was used to determine the levels of endogenous PARP mRNA. Control or antisense cells were incubated with dexamethasone for 0, 24 or 48 h. Total RNA was then isolated and analyzed by RPA with a 588-bp probe complementary to the 3¢ region of endogenous PARP mRNA. In control cells, there as no reduction in the endogenous levels of PARP mRNA on incubation with dexamethasone.

In antisense cells, a prominent 588- and 578-bp mRNA doublet as protected at time 0. After exposure of cells to dexamethasone for PARP mRNA was almost undetectable in both antisense clones tested, even though there was no reduction in the level of a control probe, indicating the selective elimination of the endogenous PARP transcripts.

Depletion of endogenous PARP protein in transfected cells--We next determined whether keratinocytes expressing the exogenous antisense gene became depleted of endogenous PARP protein. We previously established and characterized a HeLa cell line stably transfected with this inducible PARP antisense RNA expression vector. Consistent with the known *in vivo* stability of PARP (half-life of at least two days), 48 to 72 h were required after induction of antisense RNA by dexamethasone for the abundant concentration of PARP normally present in HeLa cells to be reduced by greater than 80%. Stable clones of epidermal cells transfected with this construct were therefore treated with 1mM dexamethasone for either 24, 48, 72, or 96 h, and extracts containing equal amounts of protein ere separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins ere then subjected to immunoblot analysis with polyclonal antiserum specific for human PARP. Visualization of the membranes with enhanced chemiluminescence (ECL) revealed that the levels of PARP were significantly reduced in antisense cells after 48 h with dexamethasone. In contrast, control cells showed no reduction in the level of PARP after induction with dexamethasone for as long as 72 h (Fig. 1).

To confirm whether the reduction in immunologically detectable PARP represented a reduction in the level of endogenous PARP enzyme activity, we performed measurements on cells treated with dexamethasone for 0, 24, or 48 h. When control cells ere incubated with dexamethasone, PARP activity was reduced only slightly, so that by 48 h there was a 13% inhibition of activity. However, induction of antisense clones by dexamethasone resulted in an 80% decrease in enzyme activity within 48 h. Thus, the levels of endogenous PARP activity parallel the levels of immunologically detectable protein.

METHODS USED TO STUDY TRANSFECTED KERATINOCYTES

Biological Consequence of Reduced Levels of PARP in Human Keratinocytes. Control or antisense cells were treated for 30 min with 400 mM MNNG to induce DNA strand breaks, either with or without a 48-h pretreatment with dexamethasone. Keratinocytes were then stained with rabbit antiserum specific for PARP and guinea pig antiserum specific for poly(ADP-ribose) polymer (Fig. 2).

Double immunofluorescence of control cells revealed abundant levels of PARP either in the presence or absence of any combination of MNNG and dexamethasone, indicating the dexamethasone itself did not appreciably alter the levels of PARP in the keratinocytes. Staining for poly(ADP-ribose) polymer in control cells revealed that although very little polymer was formed in the absence of MNNG, treatment with MNNG resulted in a marked increase in the amount of polymer.

When antisense cells were not pretreated with dexamethasone, the effects of MNNG on the levels of PARP and polymer in individual cell nuclei were identical to those in control cells. However, when cells containing the PARP antisense construct were induced with dexamethasone prior to MNNG treatment, almost no PARP as detected in individual cell nuclei, and the PARP that was present was localized to distinct focal regions within the nucleus. These same PARP-positive focal areas were also positive for poly(ADP-ribose). However, the amount of polymer as markedly reduced compared with both control cells and uninduced antisense cells. Thus, the transfected keratinocytes appeared to possess the desired biochemical properties for ascertaining the function of this enzyme in skin layers.

Grafting Genetically Engineered Skin Cells. Keratinocytes derived from antisense or control clones were grown as a monolayer on tissue culture plates, and then grafted onto the backs of nude mice. To determine whether these cells could form a normal

epidermis, we killed the animals three weeks after grafting, and each of the grafts was removed and cut in half. One-half of each graft as then fixed in formalin, embedded in paraffin, and sectioned for histology, while the other half as embedded in OCT medium and quick frozen in dry ice for immunocytochemistry. Sections (6 mM) were then cut and examined. Both control and antisense keratinocytes formed an essentially normal epidermis as discerned by histological analysis. The sections showed the four compartments of normal human epidermis (the basal layer, spinous layers, granular layers, and the inoculated cornified layers), demonstrating that these immortalized human keratinocytes can undergo terminal differentiation in the graft system. To examine further the ability of these cells to differentiate terminally in the graft system, we stained frozen sections immunocytochemically with antibodies specific for human keratin 10, which is epressed in the suprabasal layers of the epidermis, and for keratin K14, which is induced at the level of transcription in the lower layers of the normal human epidermis but persists as a protein in all layers of the epidermis. The reconstituted human epidermis demonstrated a similar staining pattern for human keratins 10 and 14 to that of normal human epidermis. Thus, the normal histological and immunocytochemical pattern of differentiation is observed in the reconstituted grafted epidermis.

We then determined whether PARP antisense RNA could be induced in cells containing the antisense construct when used to form a reconstituted epidermis in the graft system. Grafting was performed as described above, and the animals were then treated either topically with 75 mg of dexamethasone in acetone at the graft site, or else injected with 200 mg of dexamethasone in sterile PBS in the carotid artery. Animals were killed at 0, 6 or 24 h after dexamethasone treatment, and grafts ere quick-frozen and sectioned as described above. The sections were then examined by *in situ* hybridization for the presence of antisense transcripts within the skin. Topical dexamethasone treatment for 24 h induced antisense transcripts within the epidermis of animals grafted with cells containing the antisense construct. Neither antisense cells that were not treated topically with dexamethasone, nor control cells that were either untreated or else treated with dexamethasone, shoed hybridization to the antisense-specific riboprobe.

As a positive control for the *in situ* hybridization technique, as well as to demonstrate that the cells within the graft site were derived from human cells and not mouse cells from the host, sections were hybridized with a riboprobe specific for the 3 prime nontranslated region of mouse keratin 1 (MK1) mRNA (this probe does not react with human epidermis). The MK1 probe hybridized only outside the periphery of the graft site, not in the region where PARP antisense RNA was detected, demonstrating that the epidermis at the graft site is derived entirely from the grafted human keratinocytes.

Treatment of Engineered Human Skin Layers with Sulfur Mustard. We set out to determine whether poly(ADP-ribose) polymer formation could be induced in the grafted epidermis in response to DNA strand-breaking agents(Fig. 3). Skin grafts were therefore derived from the control human keratinocyte lines as described above, and then treated with sulfur mustard by the vapor cup method. Animals were then killed, and frozen and fixed sections of the grafted human epidermis were prepared as before. Staining of the skin sections of skin grafts treated with sulfur mustard but not in control grafts.

During the midterm period, 24 animals (nude mice) were grafted with antisense keratinocytes. Twelve animals were grafted with clone A2-2 and 12 with clone A2-9. Six weeks after grafting, half the animals were treated with sulfur mustard vapor, and seven weeks after grafting the other half was similarly treated.

INITIAL *IN VITRO* STUDIES TO CLARIFY THE CLEAVAGE OF PARP DURING APOPTOSIS

Preparation of extracts. Cytosolic extracts were prepared from cultured human osteosarcoma cells by homogenizing PBS-washed cell pellets in 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride, 10 mg ml^{-1} pepstatin A, 20 mg ml^{-1} leupeptin, 10 mg ml^{-1} aprotinin (at $1x10^8$ cells ml^{-1}) and recovering the post 100,000g supernatant after centrifugation.

Preparation of [^{35}S] PARP Substrate. The full-length cDNA clone for PARP (pcD-12) was excised and ligated into the Xhol site of pBluescript-11 SK+(Stratagene) then used to drive the synthesis of [^{35}S] methionine-labelled PARP by coupled transcription/translation.

Assay for Apoptosis, *in vitro*. Nuclei were isolated from non-apoptotic (day three) cells essentially as described before the isolated nuclei from $2x10^6$ day-three cells were combined with 25ml of the cytosol fraction ($2.5x10^6$ cell equivalents) from cells maintained for the indicated times in culture then incubated in 100ml (final volume) of a mixture containing 10 mM HEPES/KOH (pH 7.0), 50 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 40 mM b-glycerophosphate, 1 mM dithiothreitol, 2 mM ATP, 10 mM creatine phosphate and 50 mg ml^{-1} creatine kinase.

Treatment of Engineered Human Skin Layer with Sulfur Mustard. We set out to determine whether poly(ADP-ribose) polymer formation, via its post-translational modification of nuclear proteins, could be induced in the grafted epidermis in response to DNA strand-breaking agents. Skin grafts were therefore derived from the control human keratinocyte lines as described above, and then treated with sulfur mustard by the vapor cup method. Animals ere then euthanized and frozen and fixed sections of the grafted human epidermis derived as before. Staining of the skin sections with antibody specific for polymer demonstrated that under the conditions used, polymer could be detected in sections of skin grafts treated with sulfur mustard, but not in control grafts.

During the midterm period, 24 animals (nude mice) were grafted with antisense-containing keratinocytes.

12 animals were grafted with clone A2-2

12 animals were grafted with clone A2-9

6 weeks after grafting, 1/2 the animals were treated with SM vapor

7 weeks after grafting, the other 1/2 were treated with SM vapor

INITIAL IN VITRO STUDIES TO CHARACTERIZE THE CLEAVAGE OF PARP DURING APOPTOSIS

Preparation of Cell Extracts. Cultured human osteosarcoma cells ere washed in PBS and homogenized at $1x10^8$ cells/ml in 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS detergent, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pepstatin A (10 mg/ml), leupeptin (20 mg/ml), and aprotinin (10 mg/ml). The homogenate was centrifuged at 100,000g and the resulting supernatant was used as the cytosolic fraction.

Preparation of [^{35}S]PARP Substrate. The full-length PARP cDNA as excised from clone pcD-12, ligated into the Xho I site of pBluescript-II SK+(Stratagene), and then used to drive the synthesis of [^{35}S]methionine-labeled PARP by coupled transcription and translation.

Assay for Apoptosis *in Vitro*. Nuclei ere isolated from nonapoptotic (day 3) cells essentially as described before. The isolated nuclei from 2×10^6 day-3 cells were combined with 25 ml of the cytosolic fraction (2.5×10^6 cell equivalents) from cells maintained for

various times in culture, and the mixture was then incubated in a final volume of -100ml containing 10 mM HEPES/KOH (pH 7.0), 50 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 40 mM b-glycerophosphate, 1 mM dithiothreitol, 2 mM ATP, 10 mM creatine phosphate, and creatine kinase (50 mg/ml). After 2 h at 37°C, nuclear chromatin as stained with Hoechst 33342 (5 mg/ml) and examined by fluorescence microscopy.

Recent research on apoptosis has suggested that molecular damage induced by a variety of toxic agents is not per se the direct cause of cell death. Rather, the molecular damage, such as that caused by exposure of skin cells to HD, could also provide a signal for activating a pathway leading to apoptosis. If HD exposure results in apoptosis, this would not only help explain the mechanism of HD toxicity but also might provide opportunities for therapeutic intervention.

Recently, PARP has been shown to be selectively cleaved during the early stages of apoptosis in a variety of cells by a cysteine protease with properties similar to those of interleukin-1B- converting enzyme (ICE).

Because of the interest in the relation between PARP and HD toxicity, the PI, in collaboration with researchers from the Merck Frosst Center for Therapeutic Research, identified from the five known human members of the ICE family of proteases (ICE, ICE $_{rel}$-II, ICE $_{rel}$-III, Nedd-2/ICH-1 and CPP32) the enzyme that is responsible for the cleavage of PARP and which is also necessary for apoptosis [19]. The human PARP cDNA, which our laboratory as the first to isolate, in part with funding from this project, served as a template for transcription and translation to produce a ^{35}S-labeled PARP substrate for use in a direct assay of PARP-cleaving activity in cell extracts (Fig 4).

We used human osteosarcoma cells in initial experiments because they undergo spontaneous apoptotic death. Subsequently, in recent work, e have begun to study both human keratinocytes and primary human skin cultures exposed to HD in collaboration with Dr. William Smith (USAMRICD). The osteosarcoma cell line contains substantial PARP-cleaving activity, which showed (Fig. 4) is markedly higher in extracts from apoptotic cells than in those from nonapoptotic cells (lanes 3 and 2, respectively). The cleavage site (Fig. 5) within PARP (DEVD216-G217) was identified and shown to lie between the two zinc-finger DNA-binding motifs in the NH_2-terminal region of PARP (the automodification and catalytic domains are located in the carboxyl-terminal region of the polypeptide). Because PARP must bind to DNA strand breaks to be catalytically active, cleavage at this site renders the enzyme totally inactive in the presence of the large number of DNA strand breaks

characteristic of late apoptosis. Thus, our working model now is that exposure of skin cells to HD initially results in DNA strand breakage and a consequent depletion of NAD, as previously hypothesized. However, exposure to HD (similar to - and X-rays, various alkylating agents, and topoisomerase inhibitors) subsequently results in activation of the PARP-cleaving enzyme, which we have termed apopain and shown during the midterm of this project to be derived from CPP32. The cleavage of PARP would prevent further NAD depletion in keratinocytes and induce cell death by apoptosis rather than by necrosis, which would result in an inflammatory response.

Thus, during the midterm, we also showed that, coincident with apoptosis in postconfluent osteosarcoma cell cultures, the PARP-cleaving activity measured in cell extracts increased more than 10-fold (Fig. 6). PARP-cleaving activity as maximal on day five, just prior to the extensive internucleosomal DNA cleavage (Fig. 7) that occurs in this system.

During this midterm, e also explored the sequence proximal to the Asp_{216}-Gly_{217} bond as a template for inhibitor drug design. A tetrapeptide aldehyde containing the P_1-P_4 amino acid sequence of the PARP cleavage site (DEVD216-G217) was synthesized (Ac-DEVD-CHO). In collaboration with our Merck colleagues, we showed that Ac-DEVD-CHO inhibits PARP-cleavage activity in cell extracts with a 50% inhibitory concentration (IC_{50}) of 0.2 nM (Fig. 8).

During the course of this work, a convenient *in vitro* assay (Fig. 9) for apoptosis was established, which will also be adapted for use with respect to keratinocyte toxicity induced by HD in the future experiments planned for this project. In the initial experiments, we have used nuclei from nonapoptotic (day three) osteosarcoma cells. Apoptosis-typical chromatin changes in the nuclei were measured with Hoechst 33342. Cytosol from nonapoptotic osteosarcoma cells had little effect on nuclear morphology, whereas that from progressively apoptotic cells was capable of inducing apoptosis-like changes in the nuclei (Fig. 9a). The degree of apoptotic morphology as consistent with the cellular content of apopain, as shown in Fig. 6. We showed that the peptide inhibitor Ac-DEVD-CHO, which prevents PARP cleavage, significantly reduces the ability of nuclei to undergo the morphological changes associated with apoptosis (both *in vitro* (Fig. 9b) and in intact cells (Fib. 9c), as does depletion of apopain by specific antibodies (Fig. 9d).

During future periods of this project, Ac-DEVC-CHO will be assessed for its value in modulating HD-induced cytotoxicity in keratinocytes.

MIDTERM PROGRESS ON PARP CLEAVAGE IN INTACT CELLS

Cells. Human osteosarcoma cells (American Type Culture Collection no. 11226) were cultured and apoptosis was induced as described previously [19]. Burkitt lymphoma cell line BL-30 [20] and Epstein-Barr virus (EBV)-induced lymphoblastoid cell line YB-26 were maintained [21] and induced to undergo apoptosis [22, 23].

DNA. Clone pCD12, containing the full-length human PARP cDNA in an Okayama-Berg vector, has been described [24]. This clone was used as a PCR template for construction of a PARP DNA-binding domain (DBD) expression vector. PCR was performed with (i) a 28-bp primer that contained a Bam HI restriction site upstream (nucleotides 164 to 180) and (ii) a 22-bp primer that contained a Hind III restriction site downstream (nucleotides 837 to 854) of PARP cDNA. The PARP cDNA fragment thus amplified encompassed the region that encodes the to zinc fingers of the enzyme as well as the KKKSKK nuclear localization signal. Amplification was performed for 21 cycles, and the product was then ligated into the bacterial protein expression vector pQE30 (Qiagen).

Expression of purification of the Recombinant DBD. The PARP DBD fusion protein as expressed in *E. coli* and purified to >95% homogeneity in a single step by Ni-resin column chromatography (Qiagen). SDS-PAGE revealed the size of the fusion protein to be ~30 kDa, consistent with the predicted molecular mass of the PARP DBD attached to six histidine residues. The bacterially expressed DBD was renatured and biotinylated by reaction with biotin N-hydroxysuccinimide ester at room temperature for 2 h.

Immunofluorescence and Immunoblot Analysis. Rabbit antiserum to human PARP [12] and guinea pig antiserum to poly(ADP-ribose) [25] have been described. Antibodies to PARP DBD were derived by immunization of rabbits with a peptide corresponding too amino acids 25 to 41 of human PARP. Fixation of cells, immunofluorescence, and immunoblot analysis were performed as previously described [25].

Detection of DNA fragmentation. DNA breaks were detected *in situ* with the use of a Klenow fragment-based assay system (TACS1; Trevigen). Cells were fixed and labeled with biotinylated nucleotides, with streptavidin-conjugated horseradish peroxidase and diaminobenzidine used for detection. Cells were counterstained with methyl green. Brown

nuclei were positive for Klenow labeling. DNA nucleosome ladders were observed by isolation of total genomic DNA and agarose gel electrophoresis as described previously [19].

During the midterm, we have also performed studies using immunofluorescence to follow the PARP-related biological participants through the entire time period of apoptosis. Fig. 7 shows osteosarcoma cells stained with antibodies to PARP and to poly(ADP-ribose) polymer during days 1 to 10 of spontaneous apoptosis. The amounts of both PARP and polymer increase on day three suggesting the induction of substantial chromatin reorganization or strand breaks. From days 6 to 10, during which internucleosomal cleavage (Fig. 7) and PARP cleavage by apopain (Fig. 6) occur, no poly(ADP-ribose) polymer is synthesized even though large amounts of DNA strand breaks are present. Similar results to those noted on day three might be anticipated for keratinocytes exposed to HD given the DNA strand breaking and the decrease in cellular NAD observed in these cells. It will thus be of interest to ascertain during later stages of HD treatment whether poly(ADP-ribose) is no longer generated and whether death occurs by the same apoptotic mechanism shown for osteosarcoma cells.

Also during the midterm period of this project, the DBD of PARP was cloned, expressed in *E. coli*, and purified to more than 95% homogeneity by affinity chromatography on nickel columns. To avoid the use of antibodies to detect the PARP DBD bound to DNA strand breaks in osteosarcoma cells, we conjugated the bacterially expressed DBD to biotin to allow detection with streptavidin and ECL. In preliminary experiments, we assessed the extent of DNA strand breakage in fixed intact cells by staining with this reagent. By day 10, the nuclei of many cells stained intensely (consistent with the data of Fig. 7), although many strand breaks were also detected from days 6 to 9.

This latter progress accomplished during the midterm period is one example of the ability of our basic research studies to provide new methodologies for both USAMRICD and commercial enterprises to establish new approaches to examining apoptosis in skin cells and skin tissue.

As noted above, in collaboration with Dr. William Smith (USAMRICD) we have begun to investigate whether apopain activity can be detected in primary skin fibroblasts exposed by Dr. Smith to various doses of HD. Initial results, although preliminary, indicate that apopain activity appears to be induced by HD treatment. Future studies that have been initiated already will examine the time course and concentration dependency of this effect, coupled with measurements of internucleosomal DNA cleavage and other changes associated with apoptosis.

SULFUR MUSTARD, APOPAIN ACTIVITY, AND APOPTOSIS

Having characterized the events associated with apoptosis, we have investigated, during the last period, whether any or all of these events take place after exposure of keratinocytes to sulfur mustard. Our initial experiments were performed with both cultured keratinocytes and keratinocytes grafted onto the skin of nude mice. Treatment of either cultured keratinocytes of the grafted epidermis with sulfur mustard resulted in a rapid induction of poly(ADP-ribose) synthesis as determined by immunofluorescence and immunoblot analysis. In cultured keratinocytes, polymer was synthesized within 2 h of exposure to sulfur mustard.

We next measured apopain activity *in vitro* with ^{35}S-labeled PARP as a substrate. Incubation of keratinocyte extracts with labeled PARP revealed additional nonspecific proteases not present in the other cell lines examined. We therefore took advantage of the specific peptide aldehyde inhibitor Ac-DEVD-CHO to determine which of the PARP cleavage products was specific for apopain. An Ac-DEVD-CHO-sensitive band of 89 kDa was produced after treatment of cells with either 10 or 100 mM sulfur mustard, although not to the same extent as in cells treated with known apoptotis-inducing agents.

These results suggest that sulfur mustard does not induce apopain activity to as marked an extent as occurs during apoptosis. In the absence of PARP cleavage, poly(ADP-ribose) may be synthesized until NAD and ATP are depleted and the cells become necrotic.

To confirm our *in vitro* PARP cleavage results, we tested the ability of sulfur mustard to induce PARP proteolysis *in vivo*. We took advantage of the specific antibodies to the DBD of PARP. Unlike apoptosis-inducing agents, sulfur mustard did not induce the production in keratinocytes of a 24-kDa protein recognized by these antibodies. We also used antibodies that recognize both the full-length PARP protein as well as the 89-kDa COOH-terminal cleavage product. Immunoblot analysis again revealed that, unlike apoptosis-inducing agents, sulfur mustard did not induce PARP cleavage in intact keratinocytes.

During the midterm, we also investigated whether apopain itself was generated during exposure of keratinocytes to sulfur mustard. During apoptosis, the 32-kDa proenzyme is cleaved into active forms of 17 and 12 kDa [19]. Sulfur mustard induced

conversion of <10% of the proenzyme to the active forms of apopain. Thus, although sulfur mustard induces events associated with the initial stages of apoptosis, including poly(ADP-ribose) and p53 synthesis, the lack of marked apopain activation allows PARP to remain active and to deplete the cell of the NAD and ATP that are required for the completion of the apoptotic program.

CONCLUSIONS

• To understand the role of PARP in maintenance of the epidermis, we have developed a model system in which this enzyme can be selectively depleted by induced synthesis of antisense RNA. Thus, human keratinocytes were stably transfected with human PARP cDNA in the antisense orientation under the control of an inducible promoter. Induction of PARP antisense RNA in cultured cells selectively reduced the amounts of PARP mRNA, protein, and enzyme activity.

• When keratinocyte clones containing the antisense construct or empty vector were grafted onto nude mice, they formed histologically normal human skin. The PARP antisense construct was also inducible *in vivo* by the topical application of dexamethasone to the reconstituted epidermis. In addition, poly(ADP-ribose) synthesis was induced *in vivo* by the topical application of a DNA-alkylating agent to the grafted skin layers.

• A labeled substrate was produced to measure PARP-cleaving activity (apopain) in osteosarcoma cells.

• Apopain activity was shown to be an early marker of apoptosis and reached a maximum during mid-apoptosis, prior to internucleosomal DNA degradation, in osteosarcoma cells.

• Apopain cleaves PARP at Asp_{216}-Gly_{217}, releasing the DBD from the remaining portion of the enzyme and thereby rendering it inactive and unable to consume NAD in the presence of DNA strand breaks.

• A specific tetrapeptide inhibitor of apopain (Ac-DEVD-CHO) was synthesized and shown to inhibit PARP cleavage *in vitro* at subnanomolar concentrations. This agent

inhibited apoptosis in an *in vitro* assay with naive nuclei and cytosolic extracts prepared from cells undergoing the late stages of apoptosis.

• The tetrapeptide inhibitor also, at higher concentrations, inhibited apoptosis induced by alkylating agents in intact cells.

• A new histological staining method based on biotinylated bacterially expressed PARP DBD was developed to detect DNA fragmentation.

• In preliminary experiments, PARP-cleaving activity (apopain) was detected in primary human skin cells exposed to HD.

• In human keratinocytes stably transfected with PARP antisense cDNA, apopain activity was induced by an apoptosis stimulator, tumor necrosis factor.

• Immunofluorescence studies with antibodies to poly(ADP-ribose) and either cultured keratinocytes or grafted epidermis detected rapid polymer synthesis in response to sulfur mustard.

• Apopain activity, measured as cleavage of [^{35}S] PARP, was detected in extracts of keratinocytes treated with either 10 or 100 mM sulfur mustard, although in much smaller amounts than present in cells treated with inducers of apoptosis.

• Unlike inducers of apoptosis, sulfur mustard did not induce marked conversion of CPP32 to apopain.

LEGENDS

Fig. 1. PARP Antisense Induction Lowers Endogenous PARP mRNA. Cultures were incubated in the presence of 1 mM dexamethasone for the indicated time periods (in hours). Total RNA was isolated and analyzed by a ribonuclease protection assay, using the PARP mRNA-specific probe (Materials and Methods). The 588 bp protected fragment indicates the presence of endogenous PARP mRNA. 36B4 represents the endogenous human ribosomal protein mRNA used as an internal control.

Fig. 2. PARP Antisense Reduces Response of Keratinocytes to MNNG.. Cells were induced with 1 mM dexamethasone 48 h prior to MNNG treatment. Control cell nuclei not treated with MNNG express PARP enzyme (A; green), but not polymer (B), and appear green in the double exposure (C). Control cells treated with MNNG express PARP enzyme (D; green) and polymer (E; red). Colocalization of PARP and polymer appears yellow in the double exposure (F). G-I: PARP antisense cells treated with MNNG. *Bar*, 0.1 mM for all panels (magnification x 80).

Fig. 3. Topical Sulfur Mustard Induces PARP Polymer in Reconstituted Human Epidermis. Grafted human epidermis was treated topically with sulfur mustard for eight min, and the grafts embedded in OCT medium and quick-frozen. Sections of untreated (top) or treated (bottom) grafts were then analyzed by indirect immunofluorescence, using antibody specific for PARP polymer (Materials and Methods). *Bar*, 0.05 mM for both top and bottom panels (magnification x 630).

Fig. 4. PARP-cleaving activity in spontaneously apoptotic osteosarcoma cells. [^{35}S]PARP was generated by *in vitro* transcription and translation, and then combined with cytosolic extracts from preconfluent, nonapoptotic osteosarcoma cells (4.5 mg, lane 2), postconfluent, apoptotic osteosarcoma cells (4.5 mg, lane 3), freshly isolated THP-1 cells (30 mg, lane 4) THP-1 cell extracts activated by preincubation for 60 min at 37^{o}C (30 mg, lane 5), or apoptotic chicken S/M extracts (0.6 mg, lane 6). (Taken from reference [19]).

Fig. 5. Structure of PARP and the fragments resulting from its proteolytic cleavage. (Taken from reference [19]).

Fig. 6. Increase in PARP-cleaving activity in progressively apoptotic osteosarcoma cells. Cytosolic fractions were isolated from cells and then assayed for PARP-cleavage activity

(circles) or pro-interleukin-1B (proIL-1B) cleavage activity (squares) as a control. (Taken from reference [19]).

Fig. 7. Internucleosomal DNA cleavage in progressively apoptotic osteosarcoma cells. Osteosarcoma cells were maintained in culture for the indicated number of days and then collected. DNA was extracted and resolved on agarose gels. An asterisk indicates the time point (day six) at which confluence was reached. (Taken from reference [19]).

Fig. 8. Inhibition of apopain by Ac-DEVD-CHO. The cytosolic fraction from apoptotic osteosarcoma cells was incubated with [^{35}S]PARP in the presence of the indicated concentrations of the tetrapeptide aldehydes Ac-DEVD-CHO (open circles, inset) or Ac-YVAD-CHO (filled squares), which were modeled on the P_1-P_4 amino acids of the PARP cleavage site and pro1L-1b cleavage site, respectively. (Taken from reference [19]).

Fig. 9. *In vitro* apoptosis and selective inhibition by Ac-DEVD-CHO or by depletion of CPP932-mediated PARP cleavage activity. (a) Cytosol from progressively apoptotic osteosarcoma cells confer apoptotic changes upon healthy nuclei from nonapoptotic cells. (b and d) Attenuation of *in vitro* apoptosis by inhibition or depletion of CPP932. (c) Inhibition of camptothecin-induced apoptosis of osteosarcoma cells by Ac-DEVD-CHO. (Taken from reference [19]).

Fig. 10. Time course of PARP vs. poly(ADP-ribose) levels during apoptosis of human osteosarcoma cells. Cells were cultured for the indicated times, fixed, and stained with antibodies to PARP (left) or to poly(ADP-ribose) (right).

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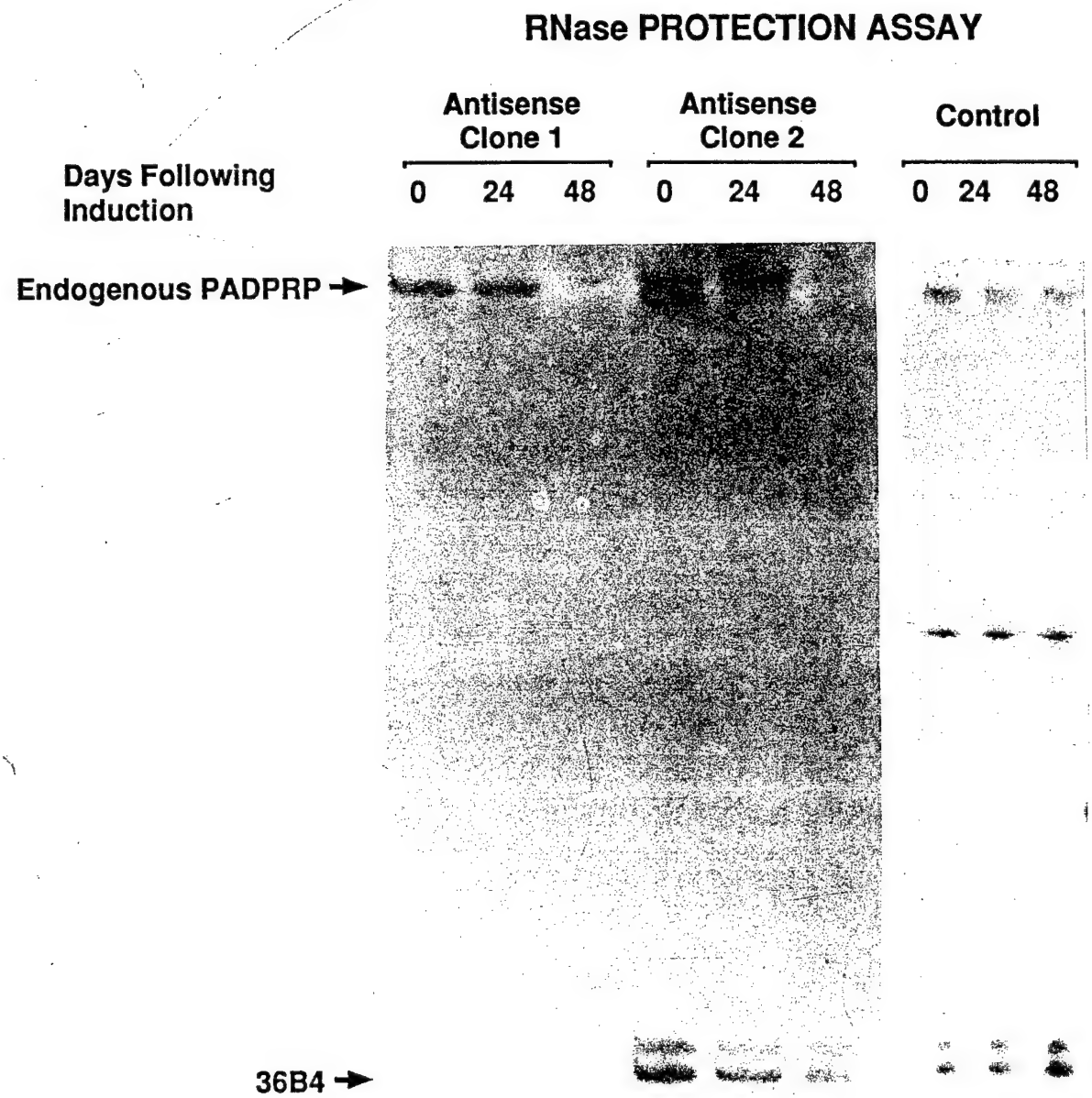
RNase PROTECTION ASSAY
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Antisense Clone 2
Control
Days Following Induction
0 24 48 0 24 48 0 24 48
Endogenous PADPRP
36B4

FIGURE 1

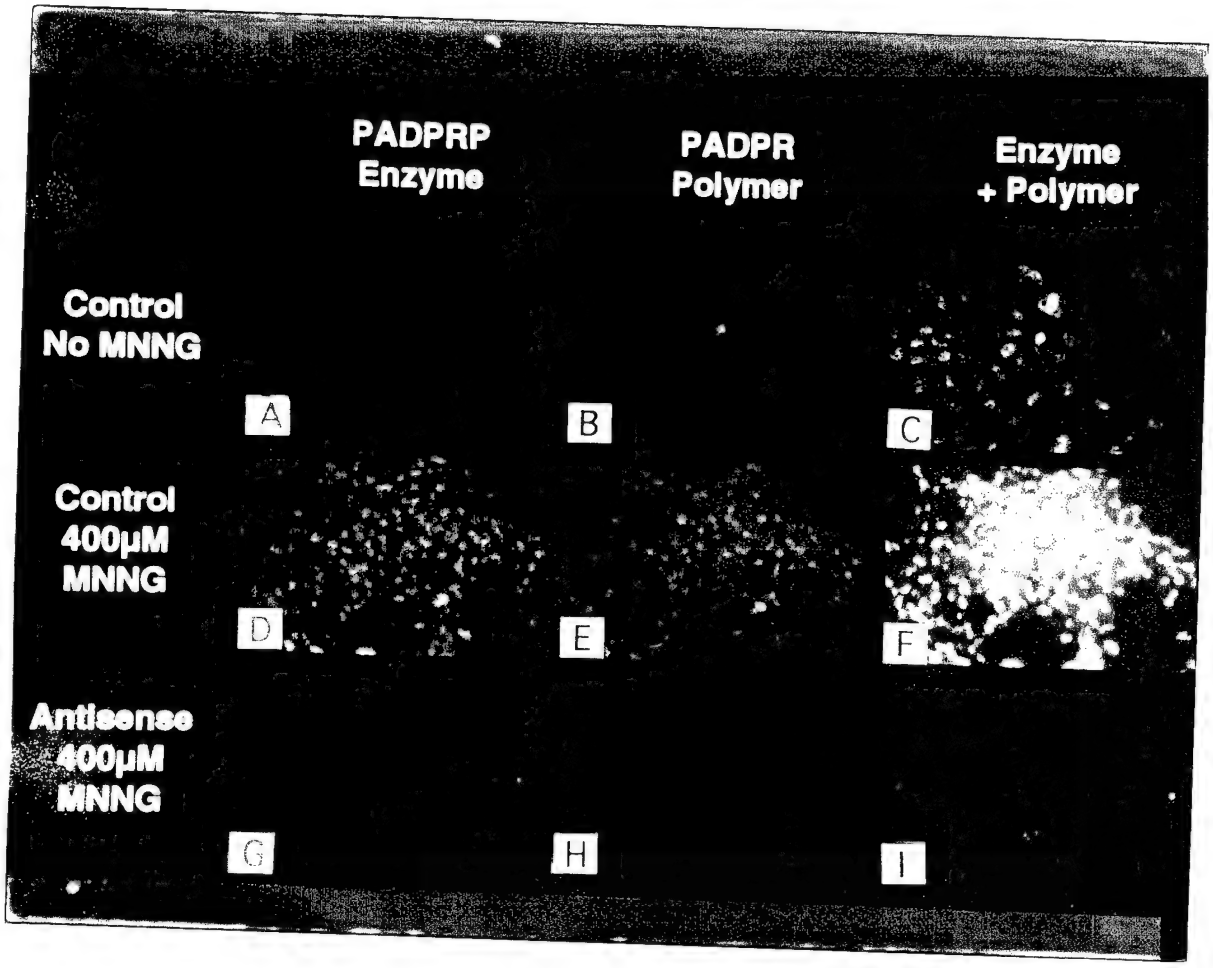
PADPRP
Enzyme
PADPR
Polymer
Enzyme
+ Polymer
Control
No MNNG
Control
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FIGURE 2

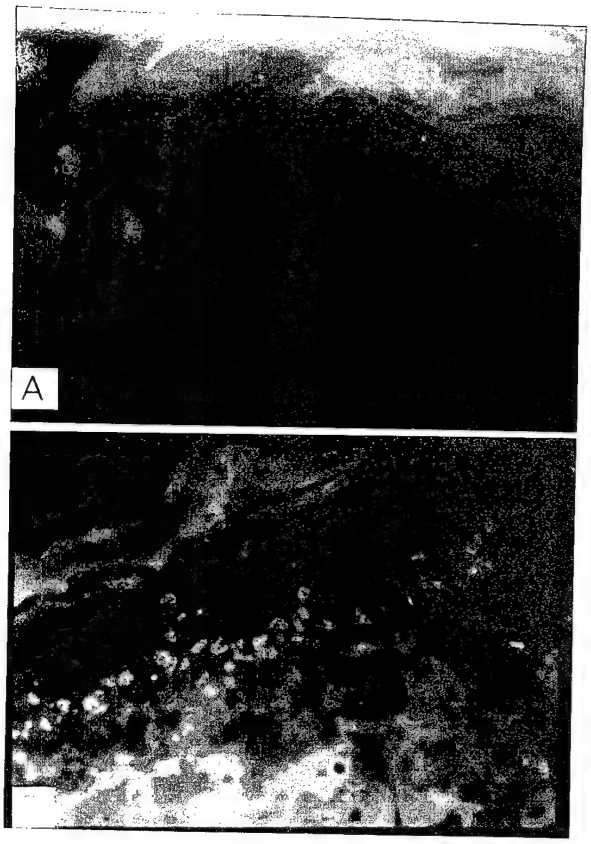

FIGURE 3

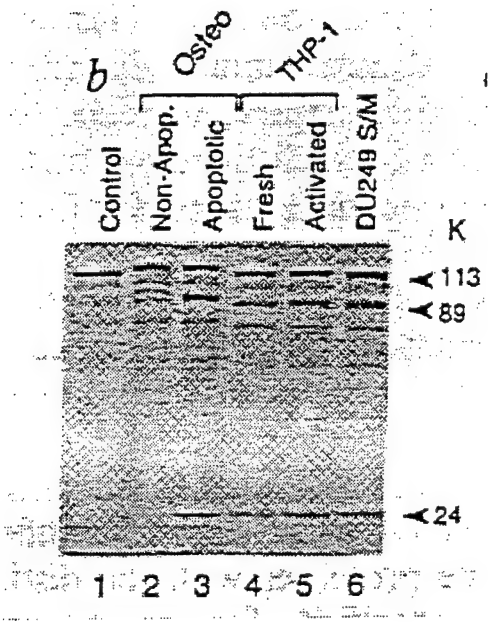

FIGURE 4

PARP cleavage activity in spontaneously apoptotic osteosarcoma cell

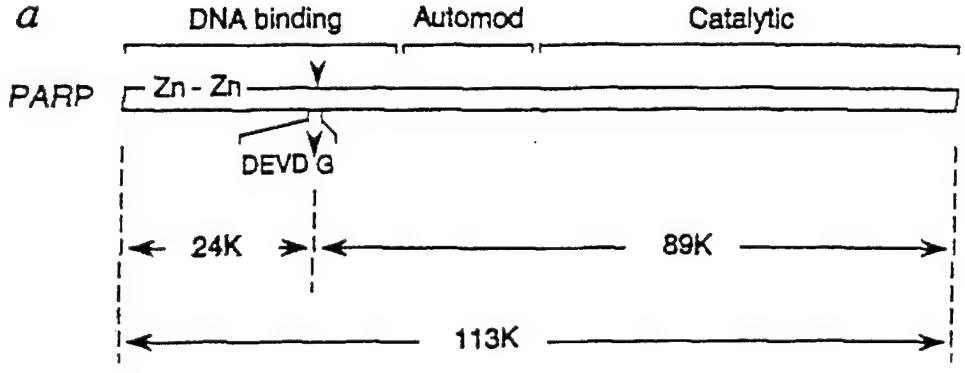

FIGURE 5

Structure of PARP and fragments resulting from proteolytic cleavage

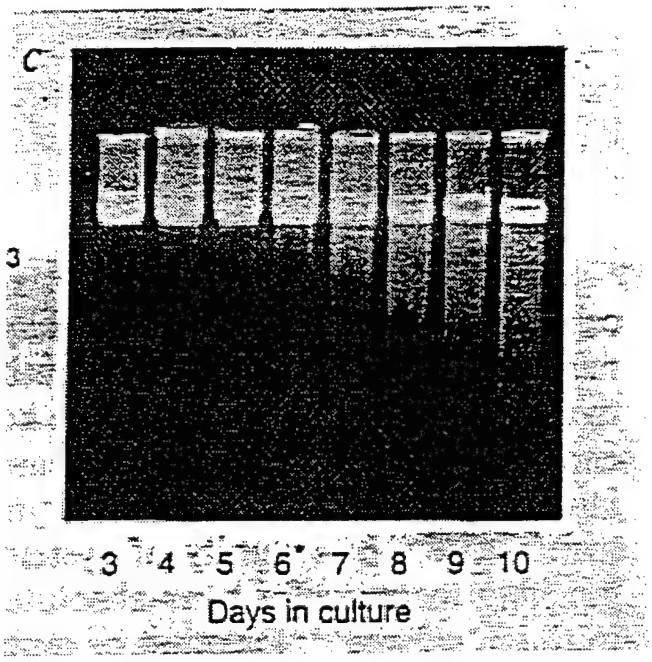

FIGURE 6
Internucleosomal DNA cleavage
in progressively
apoptotic osteosarcoma cells

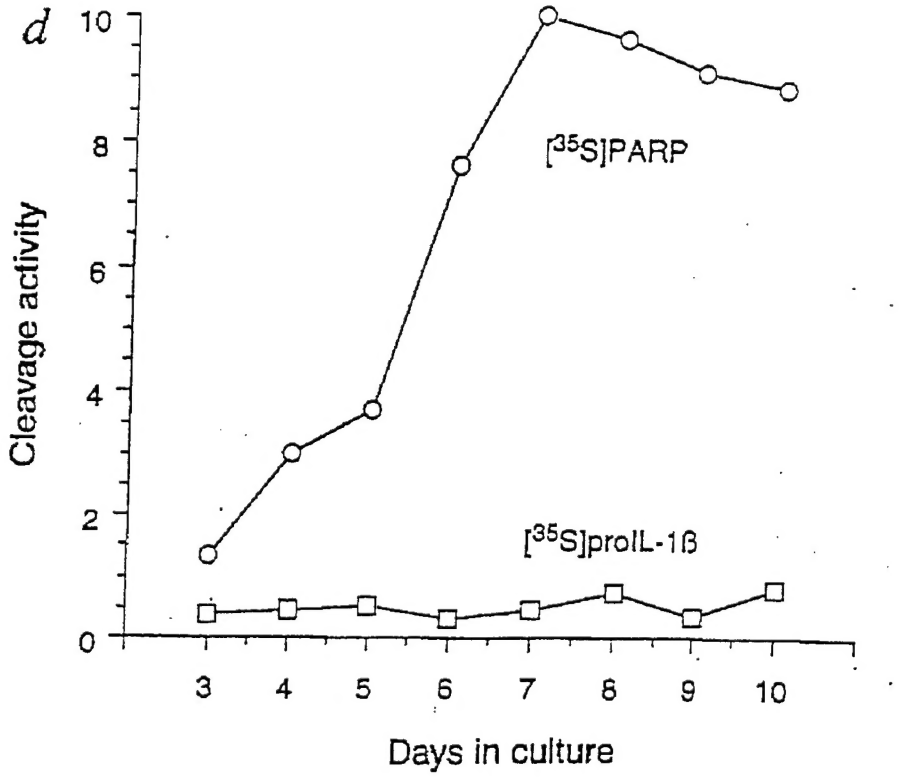

FIGURE 7

Elevation of PARP cleavage activity
in progressively apoptotic osteosarcoma cells

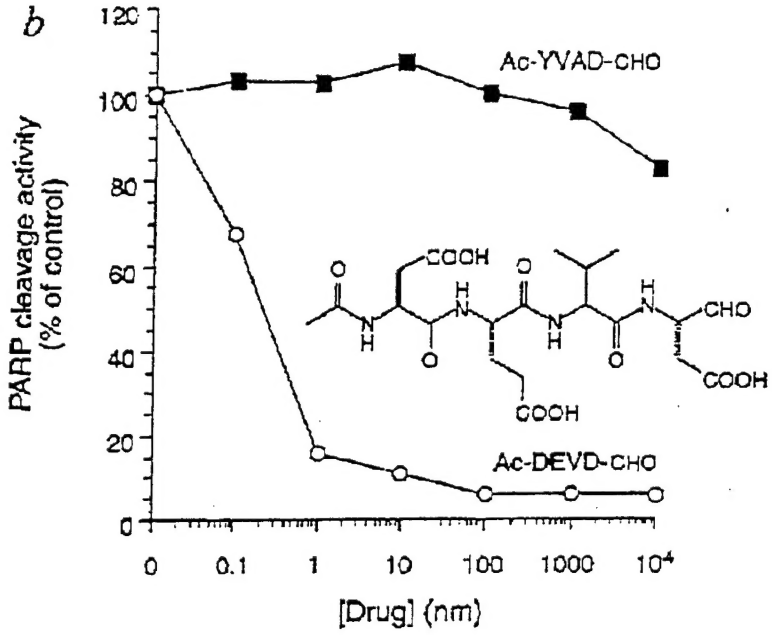

FIGURE 8
Inhibition of
Apopain by Ac-DEVD-CHO

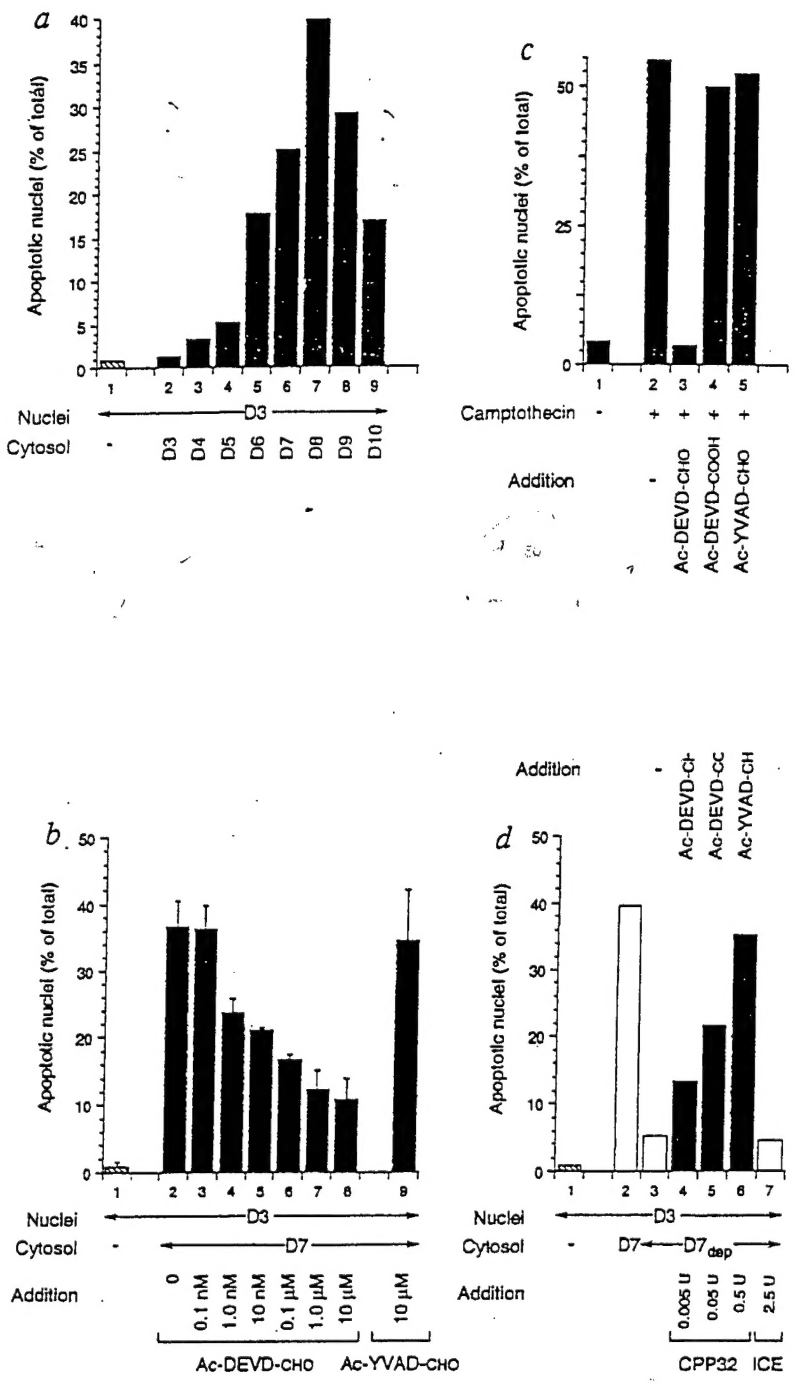

FIGURE 9

In vitro apoptosis and selective inhibition
by Ac-DEVD-CHO or by depletion of CPP-32-mediated PARP cleavage activity

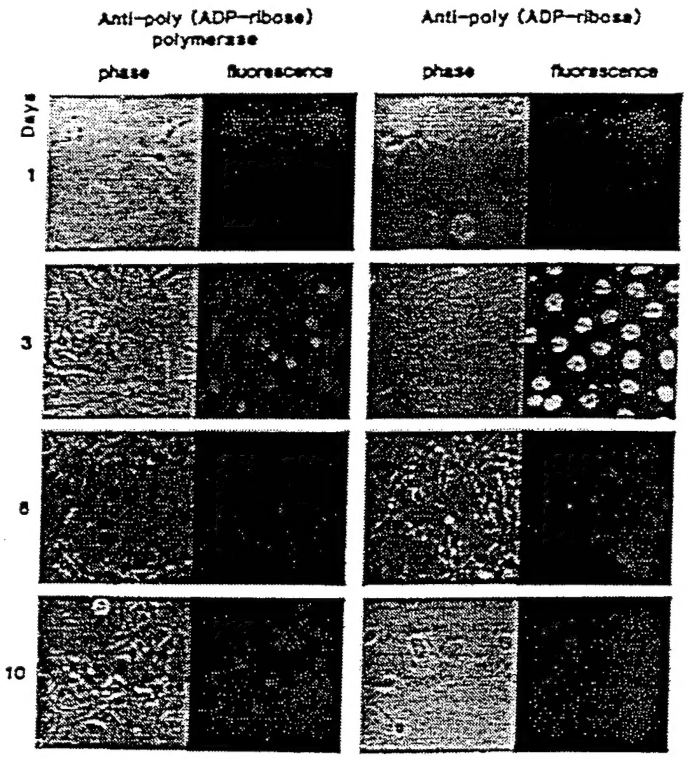

FIGURE 10

Time course of PARP vs. PAR levels during apoptosis of human osteosarcoma cells